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Assessment of Pathogenicity in *Helminthosporium maydis* causing Southern Corn Leaf Blight Disease in the Region of Karnataka

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ABSTRACT

Maize (*Zea mays L*) is the one of the most beneficial crops, adapted to various ecological and climatic states, it grades third after wheat and rice. Based on the research determinations for the last few years under the leadership of All India Coordinated Maize Improvement Project, 16 out of 61 diseases harmfully affecting this crop. One of the major diseases is Southern corn leaf blight (SCLB). The causative agent of the prevalent was recognized as the fungus *Helminthosporium maydis*. Research was carried out for pathogenicity assay. Pathogenicity assay was conducted with two methods, by collecting spores (2X10^5/ml), spraying on one month old maize plant. After 24 – 48 hours, it was found that spores collected from Davanagere (HMS3) and Kodagu (HMS5) region shows more yellow to brown lesion compare to all other regions. Second by extraction of toxin by methanol – chloroform method, purification by adsorption on charcoal and separated by using column chromatography and by thin layer chromatography. The R_f values, FTIR and UV absorption spectrum of purified toxin reveals the production of host specific toxin by H.maydis. Determination minimum toxic concentration required to satisfy the conditions as a host specific toxin.

Keywords: Survey, Pathogenicity, Extraction, Host specific Toxin, Southern Corn Leaf Blight.

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INTRODUCTION

Southern Corn Leaf Blight (SCLB) caused by *H. maydis*. This disease primarily develops serious effect to maize plant when maize crop grown under very humid and warm regions [1]. SCLB has now revolved out to be the most pervasive and serious ailments in, China, Philippines, Indonesia, Nepal, Kampuchea, Pakistan, Vietnam and India. In India it is well identified as 'Maydis Leaf Blight' and crops influenced by this condition are Corn (*Zea mays*) and Sorghum.

In India, SCLB has now turned out to be not unserious disease especially in, Himachal Pradesh, Meghalaya, Andhra Pradesh, Punjab, Haryana, , UP, Jammu and Kashmir, Bihar, MP, Sikkim Gujarat, Delhi, Maharashtra, Karnataka ,Tamil Nadu and Rajasthan. The disease is major in warm temperature ranges from 20-30^{o}C mild to tropical district. This infection has incredible enormity in the historical background of agribusiness due to its commendations in 1970 in US and consequential pulverization of a large portion of the corn edit that year. It will be in general constrained by temperature and atmosphere to the warmer parts of the US [2].

Formation spore is affected by temperature [3]. Infected tissues are broadly secured with spots and chlorosis rendering them non-profitable. It is establish to have advanced saprophytic capacity and subsequently high vital inoculum level will almost certainly be found in zones with great infection event. SCLB infection causes the critical yield calamities in cultivars created from subtropical or calm germplasm profitable from 9.7% to 11.7% rely on the climate conditions[4].

In SCLB, there are existence of two races of *H. maydis, one* is race O and another race T, are in responsibility of causing this disease in Pakistan, while race C has been accounted in China. In 1970's a pestilence caused by race T type in maize which consists of Texas male sterile germplasm in most maize emerging zones of the USA however maize with ordinary cytoplasm was resistant to the pathogen [5].

Pathogenicity assay can be led by a method of Virulence test. These approaches are significant resources to scrutinize pathogenic fungi and the effort of this technique advances in consequently [6]. To study and assessment *H. maydis* severity, a precise, intense, speedy, suitable and reproducible pathogenicity assay is necessary. The regular examination includes the inoculation of fungi by dispersal or pouring its spores or mycelia fragments[7]on a live maize leaves[8].Or else,

the leaves were immersed into uniform suspension of mycelia in a course to attain widespread lesions [9].

Plants diseased with fungi are currently notorious to produce essentials with selective toxicity in divergence to susceptible hosts[10].Several of these Host-selective toxins have been confined and partially characterized and assessed for high score pathogenicity. A genus *Helminthosporium,* a plant pathogen produces abundant host-specific toxins. A toxin which is have explicit and is accomplished to produce disease symptoms without the presence of the pathogen is named as pathotoxin [11].

The first sign of a toxin production from cultures of *H. maydis* was published in 1949 [12]. Culture filtrate set up to heat confirms to exhibits a toxin, destroying effect on maize seedlings. Isolates of *H. maydis* produces a toxin in culture can cause blight were specified as a pathotoxin. The Toxin extract from blighted plant were also accomplished to prevent root elongation progression in maize seedlings and advances a yellow lesion which are susceptible to leaf mass [13].

In 1925, it was well-defined as *Cochliobolus heterostrophus* a pathogen of maize was a heterothallic ascomycete [14]. Fungal pathogen was designated as race O to distinguish from race T, classified by revealing virulence to maize with Texas male sterile (T) germplsm in 1970. In 1969 Race T gives the impression first in the United States [15].

Hooker *et al.* discovered that race T develops a T toxin [16]. The crude extract of toxin constrains root elongation in Tms lines of maize but not N cytoplasmic lines at definite concentrations [17]. This paper discusses the prevalence of southern corn leaf blight in the region of Karnataka and pathogenicity assay of *H.maydis* causes southern corn leaf blight disease in maize along with Extraction of *H. maydis* race T pathotoxin to check the pathogenicity by root inhibition assay on seeds of N inbred and hybrid of *Z. mays.*

MATERIALS AND METHOD

Preparation of suspension culture

Potato dextrose broth was prepared and inoculated with *H. maydis* isolated from infected maize in different regions of Karnataka. The broth was incubated at 25° C for 15 days, for development of suspension culture.

Collections of spores and Assay of pathogenicity

H. maydis isolated from eleven different regions were inoculated on 50 ml of PDB in Erlenmeyer flask and were incubated at 25°C for 8-10 days. The culture was filtered to obtain the mycelia suspension and it was smashed and 100 ml of sterile water was added and shaken by hands for 5 minutes. The culture filtrate was filtered using 2-3 layers of muslin cloth to separate conidiospores from other materials. The filtrate was checked under Haemocytometer to number of spores/ml. Maize plants were grown in pots filled with sterilized sand and was positioned in control temperature room at 25°-28°C.The four weeks old plants were sprayed with 4 ml of the spore filtrate and were covered with polythene bags and it was kept in dark for 12 hours and in light for 12 hours. Every one hour the plants were sprinkled with water to keep the moisture content.

Data Analysis

The inoculated leaves were evaluated for outer symptoms at seven days interval, for duration of three weeks after inoculation (WAI). Using scoring scale [18], outside symptoms were recorded and scored for 0.5 to 5 classes of disease scale and calculated as disease severity index percentage (DSI%). Disease incidence and area under disease progressive curve (AUDPC) also used as other parameter to enumerate pathogenicity of *H. maydis.*

A total of 11 treatments (isolates and control) were tested. Disease incidence (DI) and disease severity index (DSI) was deliberated using a formulas[19, 20]. The AUDPC was calculated using a formula. [21]

Extraction and partial purification of toxin

Methods similar to those used by Pringle & Schaffer were used to partially purify the toxin[22]. The fungus was removed from the medium by straining through muslin cloth or filtering through Whatman filter papers. Original volume of the filtrate was concentrated to one tenth under vacuum at 35° C, an equal volume of methanol was added and kept at 4°C for 24hr. Methanol was removed from the supernatant by evaporation. Add three successive 10ml volumes of chloroform. After evaporation of the chloroform, the oily substances formed was weighed and dissolved in small quantities of methanol and the volume was adjusted with water to 10% of the original volume of the culture filtrate. Precipitate obtained was highly active in toxin bioassay.

The Toxin preparation was partially purified with 3% activated charcoal cooled to 4° C and filtered through Whatman filter paper. Toxin was desorbed by washing filter paper with 200ml of 5% methanol in chloroform. Partially purified toxin complex were subjected to silica gel analytical TLC. Toxin was loaded on TLC sheet was developed by using different solvent system such as chloroform: acetic acid 98:2), butanol: acetic acid: water(4:1:1), chloroform: methanol(100:5),acetone:water(9:1),Benzene: ethanol (95:5).TLC plate was cut off approximately 1/25 and subjected to the iodine vapours. The fractions obtained on TLC plate were eluted with chloroform [23].

Determination of toxin concentration

Total of hundred seeds, each of N cytoplasm seeds and hybrid seeds of *Z. mays* were surface treated, rinsed in with distilled water, and sprouted at room temperature for 3 days on sterile glass plated covered with moist filter paper in the dark. The primary root length of sprouted seeds from N cytoplasm and hybrid seeds was measured and these seeds were transferred to petri a plate which contains 10ml of a different diluted toxin concentration and also transferred to plate contains distilled water as a control. Primary root tips were made immersed in those petriplates. The following toxin dilutions were examined, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. After every four hours of interval, with period 48 hr. at room temperature the total length of the primary root was measured. Averages length of the root at beginning of the experiment were subtracted from the averages of root lengths at the completion of the experiment to determine the average root elongation or inhibition with different dilutions of toxin. Completely randomized design (CRD) was used for these experiments and each treatment records were subjected to one way analysis of variances (ANOVA) to find the significance of treatment using Web Agri Stat Package Software (version 2.0) to compute the difference between the treatments.

Purification and separation of toxin by silica gel column chromatography and TLC

Silica gel about 60-120 mesh was used to pack the column. The silica bed was washed with 500ml of solvent before use. The toxin sample was placed on top of the column and eluted with the chloroform and methanol solvent system. About 2ml fractions of eluted samples were collected, the solvent was evaporated and the residues were dissolved in

small volume of acetone. The toxin containing fractions were further purified by thin layer chromatography.

The preparative TLC was established by using a solvent system butanol: acetic acid: water (4:1:1) as mobile phase. Around 40% silica gel as a stationary phase to perform TLC. Approximately 1/20th of the TLC plate was cut off and vaporized with iodine it gave typical yellowish colour. The fractions obtained was eluted with Chloroform and concentrated in vacuo.

Spectrometry

UV-visible spectroscopy is extensively used for the examination of groups of atoms categorized by intensely absorbing electronic transitions. Set with wavelength range 200 to 400 nm.UV-visible spectra of chloroform were examined as a blank. UV-visible spectra of toxin Band 1, Band 2 and Band 3 extract of *Helminthosporium maydis* was examined as sample to determine the wavelength of absorption. Infrared spectra were recorded from films deposited on NaCl windows from chloroform solutions.

RESULT AND DISCUSSION

Pathogenicity of *H. maydis* was examined by spraying the spores containing 2.0×10^5 spores/ml on four weeks old maize plants. After 24 – 48 hours, leaves were observed for the level of yellow to brown lesions. It was detected that primarily the yellow lesions which appeared in minor sized oval shape and this advanced to widespread leaves part covering the greenish part of leaves (Figure 1). Chlorophyll on a leaf doesn't get exposed to sun, hence, inhibiting the accomplishment of photosynthesis. This leads to injury of epidermal cells on a leaf, thus totally decreasing the reproducible capability of plants. According to the level of death of cells arisen on leaves, the rating was given out of five for all 11 samples (Table 1).

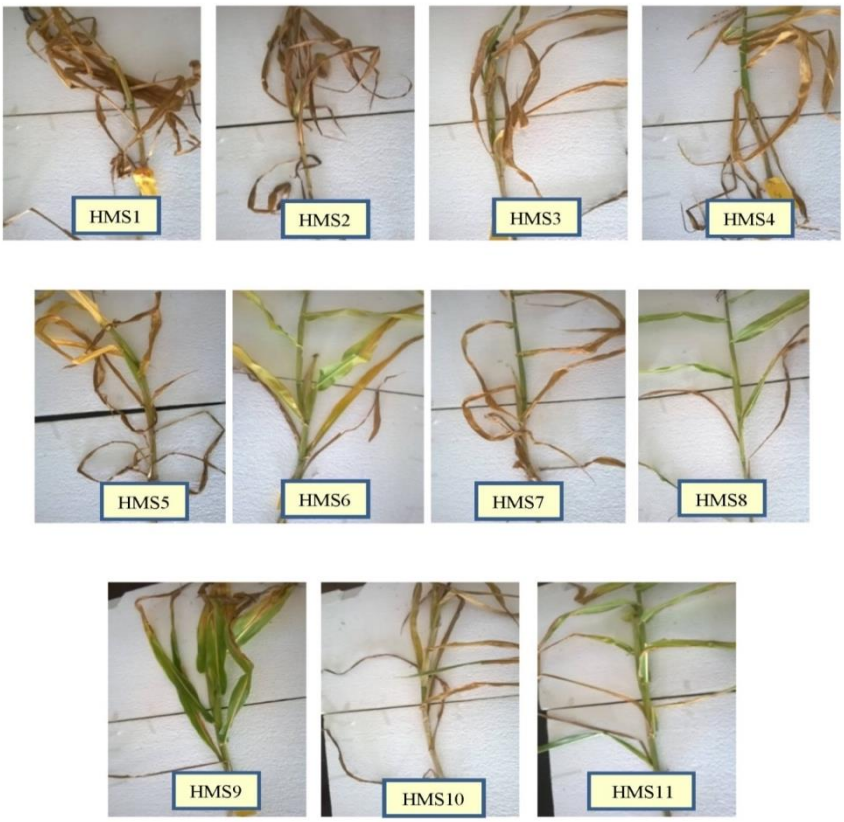

Figure 1: Eleven isolates of *H. maydis* causes yellow to brown lesions on leaves, shows a severity in pathogenicity.

Table 1: Ratings out of 5 for disease assessment in different regions

Sl. No.	Sample Code	Name of the isolates.	Karnataka region Samples	Ratings out of 5
1	HMS1	*Helminthosporium maydis*	Shivamogga	4
2	HMS2	*Helminthosporium maydis*	Chitradurga	4
3	HMS3	*Helminthosporium maydis*	Davangere	5
4	HMS4	*Helminthosporium maydis*	Tumkur	3
5	HMS5	*Helminthosporium maydis*	Kodagu	5
6	HMS6	*Helminthosporium maydis*	Mandya	1
7	HMS7	*Helminthosporium maydis*	Chamrajnagar	4
8	HMS8	*Helminthosporium maydis*	Mysore	1
9	HMS9	*Helminthosporium maydis*	Bengaluru rural	1
10	HMS10	*Helminthosporium maydis*	Chikkamagaluru	4
11	HMS11	*Helminthosporium maydis*	Hassan	1

Based on the pathogenicity score scale of *H. maydis* isolates, disease severity index (DSI) was calculated. After third week of inoculation, it was found that samples HMS1, HMS3 and HMS5 showed a highest level of pathogenicity with mean DSI % of 58.56%, 54.74% and 61.73% compared to all other isolates. While other isolates such as HMS2, HMS7 and HMS10 has shown mild level of aggressiveness when compare to HMS4, HMS6, HMS9 and HMS11 shows a lowest level of pathogenicity (Table 2 and Figure 2). Based on pathogenicity test, the symptoms of SCLB disease first appeared as brownish red spots, the lesions matured to become zonate.

Table 2: Disease severity index (%) of *H. maydis* from infected corn plants.

Isolates	1WAI%	2WAI%	3WAI%	Mean DSI%	AUDPC
HMS1	4.76	30.47	70	58.56	1140
HMS2	3.33	24.76	56.66	46.93	840
HMS3	4.28	23.80	80	54.74	1070
HMS4	1.428	9.047	40	23.80	690
HMS5	4.76	25.23	95.23	61.73	1290
HMS6	1.428	3.809	15.23	10.31	310
HMS7	2.38	17.619	58.09	39.36	860
HMS8	0.476	9.047	13.33	13.96	250
HMS9	1.428	5.238	11.42	10.47	210
HMS10	2.857	20.95	65.11	45.51	900
HMS11	0.476	5.71	13.33	10.62	210
Control	0	-	-	-	0

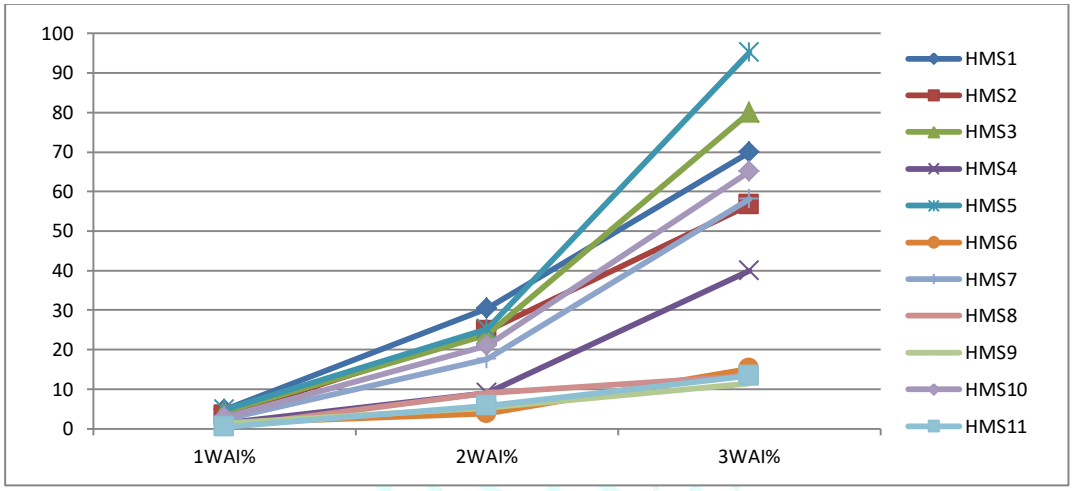

Figure 2: Mean DSI% against *H.maydis* pathogen on corn plant

In a similar incidence Mubeen et al. [24] reports, Pathogenicity of the fungal growth was examined on maize plants. Information on un-inoculated (control) and inoculated plants were being used as the signs of disease present on the leaves. Un-inoculated seedlings (control) do not display any symptoms throughout this study. Percent plant contamination and disease seriousness on un-inoculated plants were accounted. These innovations are likewise in accordance with work by Degani [25] critically proved the assertiveness of *H.maydis* pathogen. On the other hand, it was examined to acquire numerous lesions on corn leaves by suspension of mycelia[28].It was also inspected plant pathogenic fungi along with their array of growth and reproduction[26]. It was reported that evaluated *C. heterostrophus* strains found to be different in term of pathogenicity[27].

Extraction of toxin:

The practice of thin layer chromatography to identify, detect and partially purify numerous mycotoxins. Toxins were extracted from filtrates of culture by means of methanol and chloroform as a solvent. Methanol and chloroform well-known to useful solvent for the extraction of toxin. Similar reports were reported by Kono *et al.*[29] in their research on Toxin compound. Mycelium extract and Culture filtrates were purified using charcoal, the constituents was extracted using chloroform and purified by precipitation using methanol or acetone. Obtained four compound from the toxin extract, inspected their biological and physicochemical characteristics[10].

Determination of toxin concentration

Most reliable technique for determination of toxin concentration is Root elongation inhibition assay provides the results proportional to the involved concentration. The data represented in the table and the graph regarding the inhibition of primary root elongation at certain concentration and at certain time intervals.

Normal seeds and hybrid seeds treated with various concentration of toxin dilution and water as a control.10^{-1} toxin dilution shows more than 50% root inhibition can be documented to biological variation (Table3 and Figure 3, 4). While, hybrid seeds shows a moderate rate of inhibition. The rates appear to be linear over 48hrs period which indicates slowly in growth process rather than ending in death. Factor significant value is < 0.05 it means at least one treatment level is different from other and interaction effect also showing significant.

Table 3: Inhibition of primary root elongation of germinated seeds with various toxin dilutions

Toxin dilution	Primary Root length (mm)					
	Normal Seeds			Hybrid seeds		
	Initial root length (Average)	Final Root length (Average)	Net growth for 48 hrs.	Initial root length (Average)	Final Root length (Average)	Net growth For 48 hrs.
10^{-1}	9.267	9.833f	0.566	9.2	10.6f	1.4
10^{-2}	9	21.767e	12.767	9.167	20.733e	11.566
10^{-3}	8.9	29.933d	21.033	9.233	25.6d	16.367
10^{-4}	9.067	31.767c	22.73	9.267	32.433c	23.166
10^{-5}	9.333	40.767b	31.434	9.133	38.367b	29.234
H_2O Control	9.167	45.833a	36.666	9.267	45.4a	36.13

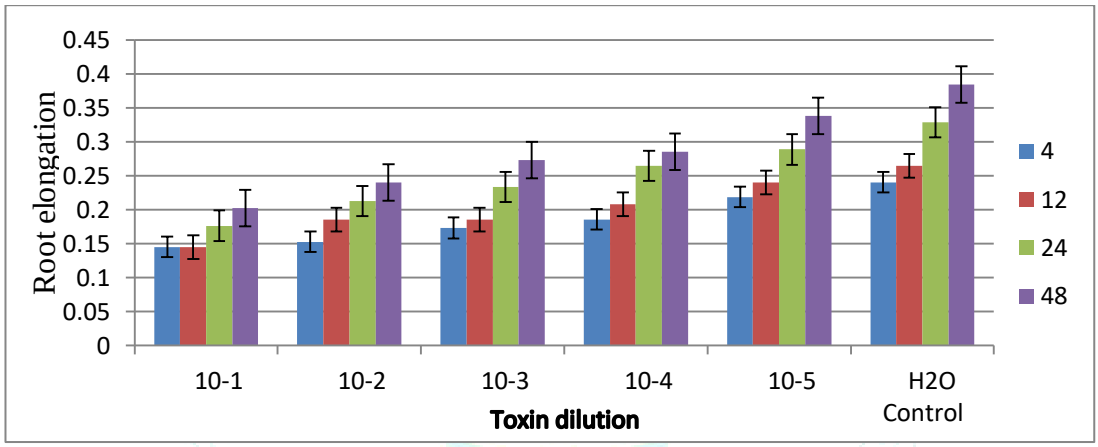

Figure 3: Inhibition of primary root elongation of N- cytoplasm germinated maize seeds

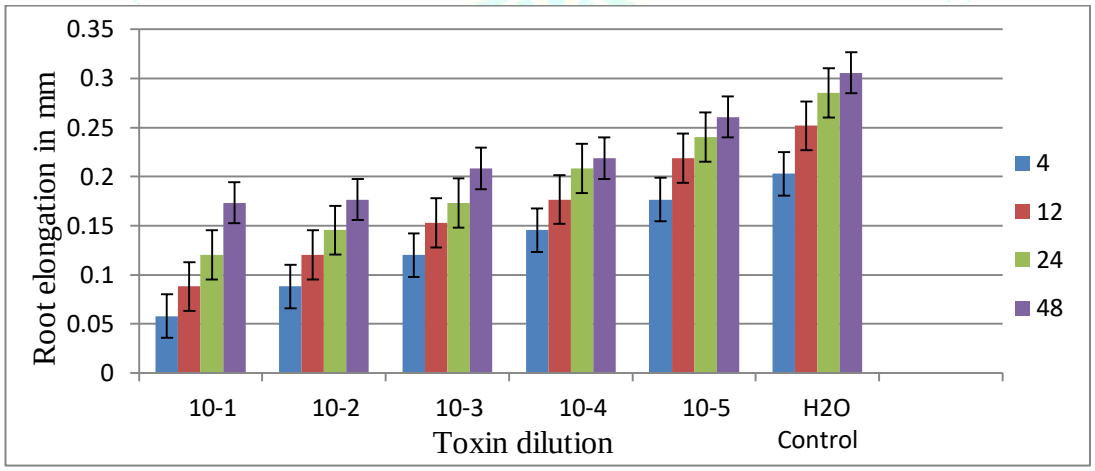

Figure 4: Inhibition of primary root elongation of germinated hybrid maize seeds at different toxin concentration

For N cytoplasm as per Analysis of variable table, for 4hrs F prob > 0.05 it means there is no difference between different levels of treatment application. For 12hrs it shows that F pro < 0.05 it means at least one treatment level is different from other. From comparison of different treatment mean with critical difference, there is no difference between 10^{-3}-10^{-2} treatments and there is no difference between 10^{-2}-10^{-1}. But for 24hrs and 48hrs are significant, F pro < 0.05 it means at least one treatment level is different from other.

For Hybrid cytoplasm as per Analysis of variable table, for 4hrs F prob > 0.05 it means there is no difference between different levels of treatment application. For 12hrs, 24hrs and 48hrs is significant, F pro < 0.05 it means at least one treatment level is different from other. Reports also achieved for extracted toxin from *H. maydis* were associated with the extension of the yellow injury on Tms cytoplasm. A Tms cytoplasm plant produces the normal yellow lesions with the toxin however not in N cytoplasm plants.

Phytotoxin achieve a significant role in host plant with pathogen interaction. Several fungi were testified to produce toxin as a secondary metabolites in to the media[30]. An another attentive experimental work also reported by Michael Thomas Turner that N cytoplasm plants are subjective by a toxin from *H. maydis* race T comparative as tms cytoplasm with connection to essential root inhibition[31].

A sample purified from silica gel column chromatography obtains fractions. Fractions are subjected to TLC results three major bands and some minor Bands. Individual toxin components separated by TLC. In solvent system toxin migrated at different R_f values. In Butanol: Acetic acid: Water (4:1:1) solvent system attains a fraction from R_f 0.2 to 0.5 and toxin compound was separated into five to six spots with

three major bands and other minor bands (Figure 5). Toxin constituents separated on TLC revealed in the region of identical biological activities [32].

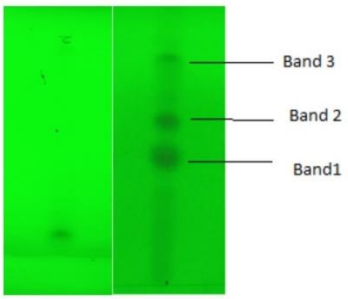

Figure 5: Toxin components separated on TLC

An attentive of test work furthermore revealed that, toxin segments was isolated into seven to nine spots on silica gel TLC by developing a dissolvable arrangement of 9:1, chloroform methanol. Isolation of four toxins from the compound, determined their physicochemical properties and recommended structures for Band 1 and Band 2-toxin are the significant parts of the toxin complex[33, 34].

Extracted toxin bands were subjected to the absorbance spectra to enquiry the presence of components, in this study it was reported that toxins I, II, and III the product eluted by using chloroform shows absorbance maximum at 275 and 300 nm. The wavelength chosen to measure the toxin, range was 330 nm. The toxin absorption was carried out on a UV spectrum at 200 to 240 nm in quartz cell. In this range, absorption showed maximum absorbance at 235.1 nm with OD of 1.707 for toxin Band 1 extract (Figure 6), 232.6 nm with OD of 1.458 for toxin Band 2 extract (Figure 7) and 228.4 nm with OD of 0.691 for toxin Band 3 extract of *H. maydis* (Figure 8).UV visible spectra of chloroform shows 287.2 nm with OD of 0.529 as a blank. An enormous variety in absorbance procedures of the reference tests not allowed utilization of wavelengths shorter than 300 nm [35].

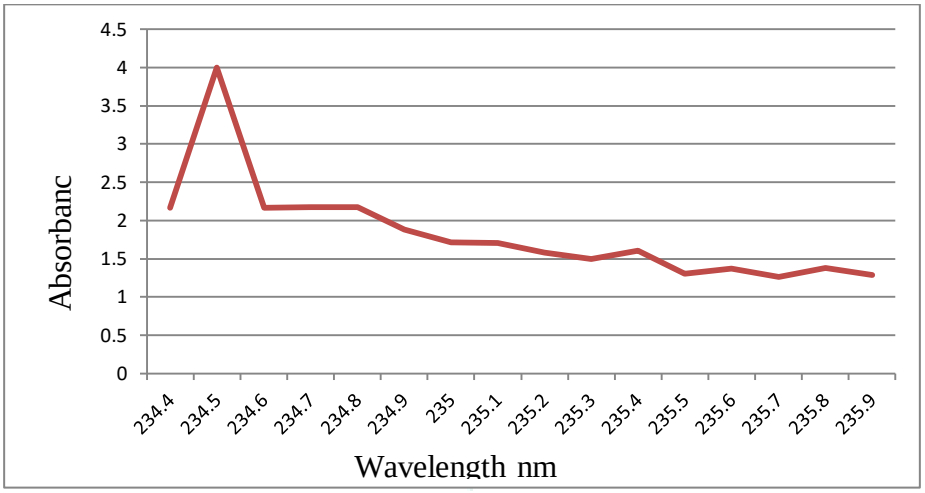

Figure 6: Ultraviolet absorbance spectra of Toxin Band1 produced by *H. maydis* eluted from TLC

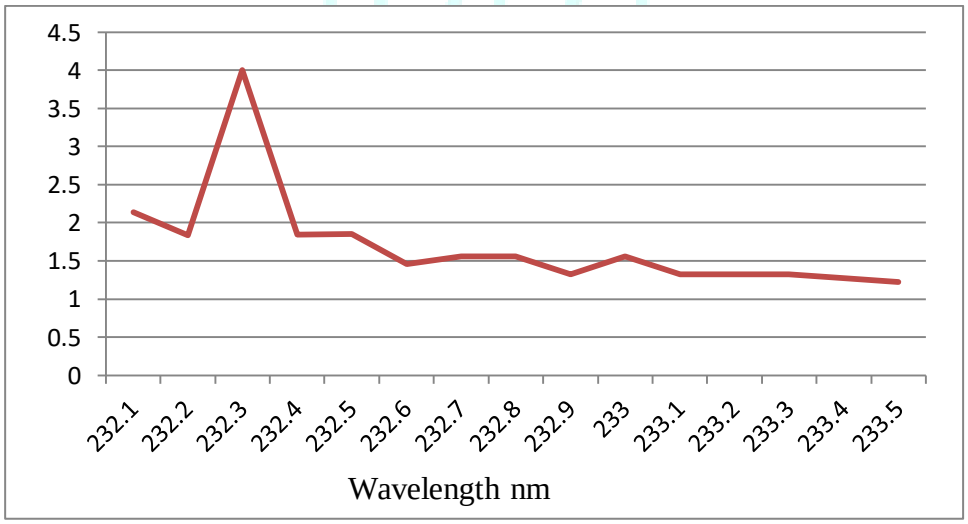

Figure 7: Ultraviolet absorbance spectra of Toxin Band 2 produced by *H. maydis* eluted from TLC

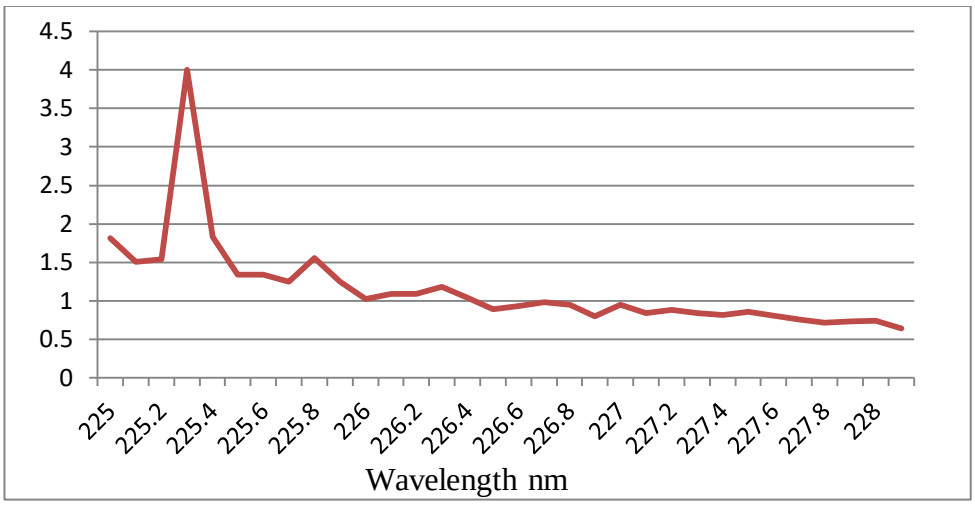

Figure 8: Ultraviolet absorbance spectra of Toxin Band 3 produced by *H. maydis* eluted from TLC

Three major bands are eluted from TLC plate by using chloroform are subjected to FTIR to find the functional group present in the residues. As such first Band results a more of C-O bond in wave number ranges between, 1255cm-1 to 1022cm-1, and C=O bond 1723 to 1590cm-1 and O-H with 3632-2555cm^{-1} (Figure 9). For Band 2 it was found to be the presence of C=O, C-O and alcohols (Figure 10). In Band 3 it was also found be C-O, C=O and the presence of alcohol as functional group (Figure 11).

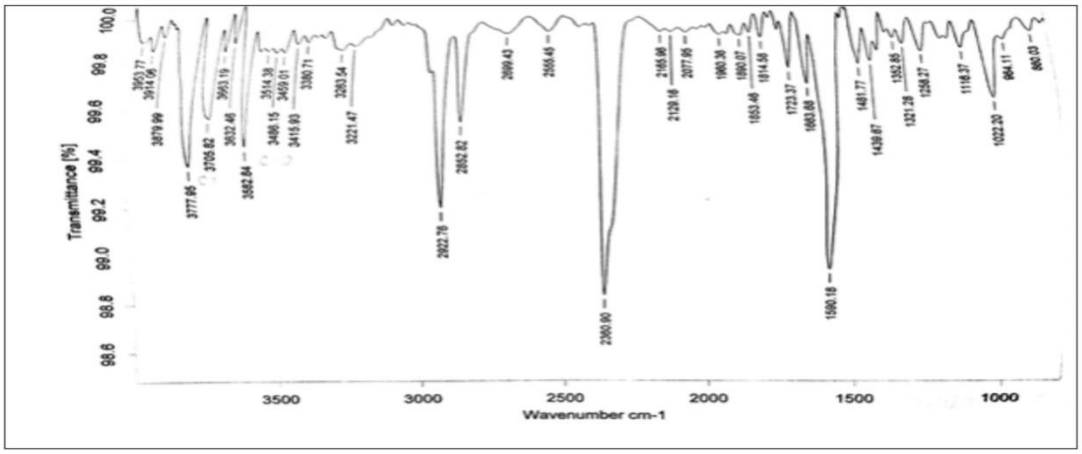

Figure 9: FTIR analysis of toxin Band 1 eluted from TLC

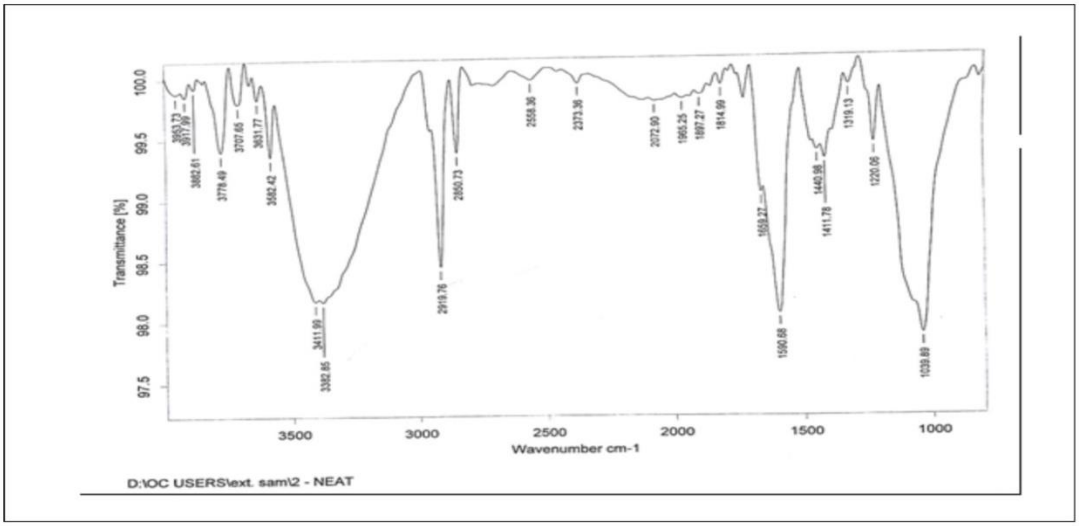

Figure 10: FTIR analysis of toxin Band 2 eluted from TLC

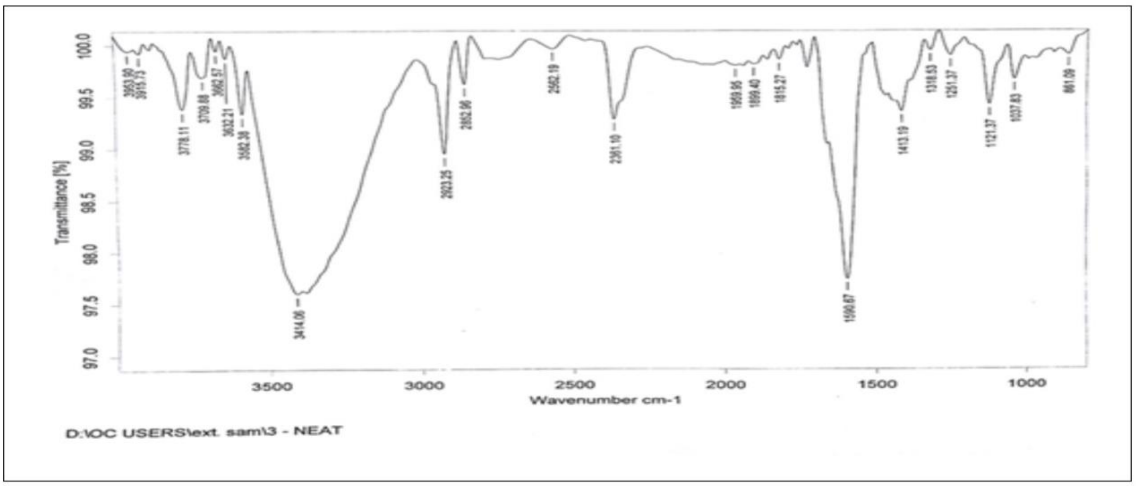

Figure 11: FTIR analysis of toxin Band 3 eluted from TLC

CONCLUSION

Survey and Pathogenicity assay reveals *H. maydis* produces secondary metabolites. Secondary metabolites are host specific toxins and virulent produced by T race of *H. maydis* in the region of Karnataka. These studies suggest that toxin from *H. maydis* are primary determinant of southern corn leaf blight disease.

Conflicts of Interest

There is no conflict of interest.

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